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(54) Title: BACTERIAL DNA PROBE

5'-GAGTTGACGTCGTCCCCGCCTTCGTC-3' (I)

5'-GAGGAAGGTGGGGATGACGTCAAGTCATCATG-3' (II)

5'-GTGGACTACTAGGGTATCTAATCCT-3' (III)

5'-ATTCCCCACTGCTGCCTCCCGTAGGAGT-3' (IV)

5'-TTACTCACCCGTTCGCCGCT-3' (V)

(57) Abstract

An oligonucleotide of formulae (I), (II), (III), (IV) or (V) or a sub-sequence thereof, is a DNA probe specific for the detection of bacteria.

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BACTERIAL DNA PROBE

In many fields testing is carried out to detect the presence of bacteria. This is important, for example, in medical diagnostics, in the food, beverage and pharmaceutical industries, in the supply of water, and in public health work in general. It may be necessary or desirable to detect not just live bacteria but also or alternatively non-viable bacteria. The testing may be carried out routinely, for example, for quality control purposes, or in the context of a specific investigation of bacterial contamination.

At present, the methods generally used include microscopy after staining, and culture and colony counting. All these methods have disadvantages, for example, there is no stain that will reliably identify all bacteria. Furthermore, non-viable organisms may lose their normal staining properties, for example, non-viable Gram positive organisms may lose their positivity. It is, of course, not possible to culture organisms that are not viable, and even with viable organisms success is critically dependant on the choice of medium. Moreover, with certain organisms in vitro culture is difficult, and for some organisms, for example, T. pallidum, in vitro culture has not yet been achieved.

A particular problem is to detect bacteria in the

pres nce of cells of higher organisms, for example, mammalian, avian, fish or plant cells. This problem is encountered especially in medical diagnostics, in the food industry and in public health work. An example of a particular problem for conventional methods (because the bacteria are non-viable) is the detection of prior bacterial contamination in food that has been treated by γ-irradiation.

A further problem area is in the diagnosis of diseases in which bacteria are believed to be implicated but the aetiology of which is unknown.

The present invention relates to a method for detecting and/or determining the presence of bacteria, either viable or non-viable.

The present invention provides a DNA probe comprising an oligonucleotide of formula I, II, III, IV or V

5'-GAGTTGACGTCGTCCCCGCCTTCGTC-3' (I)

5'-GAGGAAGGTGGGGATGACGTCAAGTCATCATG-3' (II)

5'-GTGGACTACTAGGGTATCTAATCCT-3' (III)

5'-ATTCCCCACTGCTGCCTCCCGTAGGAGT-3' (IV)

5'-TTACTCACCCGTTCGCCGCT-3' (V)

or an oligonucleotide complementary to an oligonucleotide of formula I, II, III, IV or V, (ie the corresponding cDNAs), or an oligonucleotide sub-sequence of 7 or more c ntiguous bases of an oligonucleotide of formula I, II, III, IV or V, or an oligonucleotide complementary thereto.

A DNA probe comprising an oligonucleotide of formula I or an oligonucleotide sub-sequence thereof should especially be mentioned, and also the corresponding cDNAs.

A DNA probe of the present invention is capable of hybridising with bacterial nucleic acid material substantially exclusively, that is to say, it hybridises with prokaryotic nucleic acid material but does not hybridise substantially detectably with viral or eukaryotic nucleic acid material. Accordingly, an oligonucleotide or an oligonucleotide sub-sequence thereof of the present invention can function as a DNA probe specific for the detection of bacteria. It is particularly useful to use a DNA probe of the invention to hybridise with ribosomal RNA (rRNA).

As indicated above the present invention also provides a DNA probe that comprises any sub-sequence of an oligonucleotide of formula I, II, III, IV or V, or of a corresponding complementary oligonucleotide, that retains the ability to hybridise exclusively with bacterial nucleic acid material. An oligonucleotide sub-sequence of the invention generally comprises at least 7 contiguous bases, preferably more than 7, more preferably 10 or more, and especially 16 or more contiguous bases. An oligonucleotide sub-sequence of the invention may have less than 7 contiguous bases provided that it retains the ability to hybridise exclusively with bacterial nucleic

acid material.

The present invention further provides a DNA probe which comprises an oligonucleotide of formula I, II, III, IV or V, or a corresponding complementary oligonucleotide, or an oligonucleotide sub-sequence thereof, the oligonucleotide or sub-sequence thereof having one or more base changes, rearrangements, additions or deletions with the proviso that the resulting DNA probe is capable of hybridising with bacterial nucleic acid material exclusively. A base change is especially one that results from the degeneracy of the genetic code.

Any oligonucleotide or oligonucleotide sub-sequence of the present invention may be part of a larger nucleotide with the proviso that the resulting larger nucleotide is capable of hybridising exclusively with bacterial nucleic acid material.

A DNA probe of the present invention may be produced by chemically linking the appropriate deoxyribonucleotides, as individual base(s) and/or as subsequence(s) of the desired probe, using any chemical method of DNA synthesis, see for example, Caruthers et al (1982) New Methods for Synthesising Deoxynucleotides, in "Genetic Engineering" eds Setlow J. K. and Hollaender A. pp 119-45, Plenum Press and Narang S. A. (1983) DNA Synthesis Tetrahedron 39 3-22. Solid phase synthesis is generally preferred, for example, there may be us d a solid phase synthesiser, for example, a Cyclone synthesiser made by Biosearch Inc. (N w Brunswick

Scientific Co.), the precursors used being phosphoramidites (L. J. McBride and M. H. Caruthers, Tetrahedron Lett., 24: 245, 1983).

A double stranded oligonucleotide may be produced initially by chemical synthesis and then cloned in a suitable bacterial host. Such methods have been described, for example in T. Maniatis et al "Molecular Cloning - A Laboratory Manual" Cold Spring Harbor Laboratory, New York (1982). Accordingly, the present invention also provides a DNA probe of the invention when produced by recombinant DNA technology. However, for oligonucleotides as small as those of formula I, II, III, IV and V, and the corresponding complementary oligonucleotides, and oligonucleotide subsequences thereof, chemical synthesis is generally the method of choice.

The present invention provides a method of detecting and/or determining bacteria, which comprises contacting a solid or liquid sample under investigation with a DNA probe of the invention under hybridising conditions and detecting and/or determining hybridised DNA.

The present invention further provides a DNA probe of the present invention for use as in the detection and/or determination of bacteria using a hybridisation technique.

As indicated above, the DNA probe of the present invention hybridises exclusively with bacterial

nucleic acid material, that is to say, with DNA and/or RNA from bacteria but not with nucleic acids from other life forms, for example, viruses, fungi or higher life forms. Detection of bacterial nucleic acid material may be carried out using any qualitative or quantitative method of nucleic acid hybridisation, for example, Southern blotting, Northern blotting, dot blotting or hybridisation in solution. Such methods are all well known.

Northern blotting may be carried out in the present invention, for example, as follows:

To determine bacterial nucleic acid material, especially RNA in a sample of fluid, the nucleic acid material is extracted by lysis, generally by physical or chemical means, for example, chemical lysis with guanidinium chloride or physical lysis by sonication, followed by extraction, generally with chloroform, and precipitation, generally with ethanol. The extracted nucleic acid material is bound to a solid support, for example, diazotised aminobenzyloxymethyl paper or nitrocellulose or charge-modified Nylon, for example, a nitrocellulose filter. The bound nucleic acid material is hybridised, optionally in the presence of formamide, with a probe of the invention that has previously been labelled. Specifity of hybridisation is controlled by subsequent washing of the hybridised material, for example, by the number of washes, the temperature at which the washing is

carried out, and the nature and strength of the solutions used for washing. Detection of hybridisation is carried out using a method appropriate for the label used.

Labels (also known as markers) suitable for use in detecting nucleic acid in hybridisation are known and include labels that can be detected directly, in particular radioisotopes, for example, ^{32}P, ^{35}S, ^{3}H, ^{125}I and labels that can be detected indirectly, for example, using enzymic or immunological techniques. Biotin is an example of the latter type of label, and is generally detected by methods which rely primarily on the affinity of biotin for avidin or streptavidin which may themselves by labelled or which may be reacted with a further specific reactant that is itself labelled, for example, a labelled antibody. Methods for labelling DNA are well known.

The oligonucleotide of formula I is highly specific for bacterial nucleic acid. It hybridises with all major classes of bacteria and shows no detectable hybridisation with mammalian RNA on Northern blotting. Sub-sequences of this oligonucleotide generally have decreasing specificity with decreasing size. Furthermore, the specificity of the oligonucleotide of formula I generally decreases when the oligonucleotide is incorporated in a larger molecule, the specificity in this case decreasing with increasing molecular size. For some purposes the lesser specificity of an oligonucleotide smaller or larger than that of formula I

may be acceptable, particularly if the oligonucleotide is larger or smaller than formula I by only a few bases, for example oligonucleotide sub-sequences having from 18 to 25 bases have >95% of the specificity of the oligonucleotide of formula I, which has 26 bases, and oligonucleotide sub-sequences having 16 bases still have good specificity. Below 16 bases the specificity tends to decrease, but sub-sequences of only 7 bases may be adequate for certain purposes. Generally, however, it is preferable to use a DNA probe having more than 16 bases, and for the best specificity the oligonucleotide of formula I itself is particularly preferred.

The same considerations regarding the relationship between specificity and size apply mutatis mutandis, to the oligonucleotides of formulae II, III, IV and V.

The sensitivity of the test method used can be altered by the choice of nucleic acid material to be determined and by the choice of label and detection method. There is considerably more rRNA than DNA per average bacterial cell. Accordingly, detection of rRNA is generally preferable.

Radioisotope labels are generally determined directly, and can afford qualitative or quantitative results. Other labels, for example, biotin can be detected using secondary markers. Sensitivity can be increased in some cases by

using detection techniques that involve amplification of the original label.

As suggested above, the bacterial detection test of the present invention may be carried out on any sample for which evidence of bacterial contamination is required. The samples may be already liquid or may be solid. In the latter case any bacteria or bacterial fragments present may be washed off or otherwise extracted from the sample. Many suitable methods are known, that is to say, described in the literature of the art or in actual use in the art.

The present method may also be used to determine by an in situ hybridisation method the presence of bacteria in a sample that has previously been sectioned histologically.

A sample to be analysed may be any for which the determination of bacteria, for example, in the presence of eukaryotic cells, is desired. The sample may be of a body fluid, or tissue, or any other sample of a medical nature. It may be a sample of food, beverage or a pharmaceutical undergoing analysis during manufacture for quality control purposes or it may be a sample of food or beverage undergoing analysis for suspected bacterial contamination. It will be appreciated that the present test will find application in public health work in general as well as in quality control and also research laboratories.

The present invention also provides RNA sequences that are complementary to the DNA sequences of formulae I, II, III, IV and V, and also to the corresponding cDNA sequences, and to oligonucleotide sub-sequences thereof. Such RNA sequences may themselves be useful in the detection and/or determination of bacteria, and the present invention provides such a method, which comprises contacting a solid or liquid sample under investigation with such an RNA probe under hybridising conditions and detecting and/or determining hybridised RNA probe. The invention further provides kits analogous to the DNA probe-containing kits described above, but comprising an RNA probe of the invention.

It will be appreciated that it is not generally effective to use a cDNA probe for detecting and/or determining RNA in a sample. A cDNA probe may be used to detect and/or determine DNA in a sample.

The following Example illustrates the present invention:

1. Preparation of total RNA from bacteria

Samples of bacteria were grown at 37°C on a rotary platform shaker (200rpm) to mid-log phase (optical density at 540 nm of 0.5-1.0) and 2.0 M glycine was added to a final concentration of 150 mM. After a further 16 hours of growth bacteria were harvested by centrifugation at 5000 rpm for 5 minutes in a Sorvall GSA rotor, to obtain 0.5-1.0 g wet weight of bacterial pellet. This was resuspended by gentle trituration in 15 ml of 10 mM tris-HCl pH8, 1 mM EDTA, and placed on wet ice. After 15 minutes the bacterial suspensions were sonicated with a Dawe soniprobe apparatus at 80 W, using 3 cycles each consisting of 1 minute sonication and 1 minute resting. Sonicated bacteria were then centrifuged at 10,000 rpm for 10 minutes in a Sorvall SS34 rotor, and the supernatants were decanted into glass centrifuge tubes. Phenol was prepared according to T. Maniatis et al (DNA Cloning: A Laboratory Manual; Cold Spring Harbour Laboratory, New York, 1982) and mixed with an equal volume of chloroform. The supernatants from the sonicated bacteria were mixed with equal volumes of phenol:chloroform and shaken at room temperature for 10 minutes (250 rpm on a rotary platform shaker) and then centrifuged at 7,000 rpm for 10 minutes in

a Sorvall SS34 rotor. The upper (aqueous) phase was decanted into fresh clean tubes. Extraction with phenol:chloroform was repeated two more times, and was followed by two extractions with chloroform alone. The volume of the final aqueous phase was measured, and one tenth of this volume of 4 M sodium chloride was added, followed by 2 times the final volume of ethanol. After gentle mixing the samples were stored at -20°C for at least 16 hours. Aliquots of 50-1500 µl (containing 8-10 µg of A260-absorbing material) were removed immediately prior to gel analysis, centrifuged in a Eppendorf microfuge for 2 minutes, resuspended in 1 ml 70% ethanol and recentrifuged. After removal of the supernatant, samples were dried _in vacuo_ for 5 minutes and redissolved in 5 µl of 10mM tris-HCl pH8, 1 mM EDTA.

Total RNA was obtained from _Staphylococcus aureus_, _Streptococcus_ species, _Pseudomonas_ species, _Escherichia coli_, diphtheroid, _Proteus_ species and _Mycobacterium smegmatis_ ATCC 607.

2. Preparation of total RNA from tissue samples

Armadillo liver and spleen, uninfected and infected with _M. leprae_ were obtained from the WHO armadillo tissue bank, where material is stored at -70°C. Uninfected mouse liver was obtained immediately after sacrifice of mice by cervical dislocation. Tissue samples

of 1-2 g were minced with scissors to pieces of approximately 1 mm^3, suspended in 25 ml of a 3M LiCl, 6M urea solution, and homogenised on wet ice in a Sorvall Omnimix run at full speed for 3 minutes. The resulting homogenate was kept on ice for 30 minutes to settle, and was then sonicated in a Dawe Soniprobe apparatus at 80 W for 10 cycles of 1 minute sonication, 1 minute resting. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.2% and the material was stored at 4°C overnight. RNA was pelleted by centrifugation at 7,000 rpm for 10 minutes in a Sorvall SS34 rotor and redissolved in 5 ml of 10 mM tris-HCl pH7.6, 1 mM EDTA, 0.5% SDS. This was extracted three times with phenol:chloroform and two times with chloroform as above, after which ammonium acetate was added to a final concentration of 0.4 M. After addition of 2.5 volumes of ethanol, samples were stored at -20°C for at least 16 hours. Samples were taken immediately before analysis as above.

3. <u>Preparation of labelled oligonucleotide probe</u>

The oligonucleotide probe used has the sequence;

5'-GAGTTGACGTCGTCCCCGCCTTCGTC—3'

An aliquot of 500 ng of this probe was mixed with 20 μCi of gamma-32P-ATP (5000 Ci/mmol) and 10 units of T4 polynucleotide kinase in the presence of 70 mM tris-HCl

pH7.6, 10 mM $MgCl_2$, and 5 mM dithiothreitol, and incubated at 37°C for 30 minutes. Labelled oligonucleotide was separated from unincorporated isotope by gel filtration on a Pharmacia PD-10 prepacked column previously equilibrated with, and eluted with 10 mM tris-HCl pH8, 1 mM NaCl. Peak excluded fractions were pooled, 1 mg of E. coli tRNA was added, NaCl was added to 0.3 M and the oligonucleotide was precipitated by addition of 2 volumes of ethanol with incubation at -20°C for at least 16 hours. Immediately before use, the suspension was centrifuged in an Eppendorf microfuge for 5 minutes, resuspended in 1 ml of 70% ethanol, recentrifuged, dried in vacuo for 5 minutes, and dissolved in 50 ul of 10 mM trisHCl pH8, 1 mM EDTA.

4. Northern Blotting

RNA samples from bacteria and from tissue, prepared as described in sections 2 and 3 above, were analysed on 1% agarose gels: 1 g of agarose was dissolved by heating in 50 ml of 7.2 mM tris base, 6.0 mM sodium dihydrogen orthophosphate, 0.4 mM EDTA. After cooling to 60°C, 50 ml of formamide (freshly deionised by mixing for 30 minutes with Amberlite MB-3 mixed bed resin) was added and the agarose solution was poured into a gel tray and allowed to set. Samples were further prepared by addition of tris base to 3.6 mM, sodium dihydrogen orthophosphate to 3.0 mM, EDTA to 0.2 mM, formamide to 50%, glycerol to 5% and

bromophenol blue to 0.01%, after which they were heated to 60°C for 3 minutes and cooled rapidly on wet ice. Samples were then loaded on the gel and run at 10 V/cm in 3.6 mM tris base, 3.0 mM sodium dihydrogen orthophosphate, 0.2 mM EDTA until the bromophenol blue had migrated 2/3 of the length of the gel. The gel was then soaked in 0.5 μg/ml ethidium bromide in water for 30 minutes, then in water for 30 minutes, after which it was photographed under ultraviolet light. The gel was prepared for blotting by soaking for 30 minutes in 10% formaldehyde and then for 30 minutes in 3 M NaCl, 0.3 M sodium citrate. RNA was then transferred overnight to nitrocellulose (Schleicher and Schuell type BA85) by capillary suction as described in Maniatis _et al_ (loc. cit.) using 1.5 M NaCl, 0.15 M sodium citrate as transfer fluid. The nitrocellulose sheet was then removed and dried at 37°C, after which it was baked at 80°C for 2 hours. (Gel electrophoresis was used to determine the size of the RNA used, but hybridisation can alternatively be carried out by directly blotting the samples onto nitrocellulose.)

The filter was prehybridised in a plastic bag containing 10 ml of 900 mM NaCl, 90mM tris-HCl pH7.6, 6 mM EDTA, 0.04% bovine serum albumin, 0.04% Ficoll 400, 0.04% polyvinylpyrrolidone, 100 μg/ml sonicated (to 500-1000 bp) salmon sperm DNA (heated immediately before use to 95°C for 10 minutes), and 70 μg/ml yeast tRNA, at 55°C for 3 hours. This solution was then removed and replaced with 10

ml of the same solution containing one million cpm per ml of the labelled oligonucleotide. The filter was incubated at 37°C for 2 days, after which it was washed at room temperature in a Hybaid apparatus using 1000 ml of 0.3 M NaCl, 0.03 M sodium citrate, followed by 500 ml of 0.03 M NaCl, 0.003 M sodium citrate, followed by 500 ml of 0.15 M NaCl, 0.015 mM sodium citrate. After drying at 37°C the filter was exposed to Fuji X-ray film for 16-60 hours at room temperature.

The results obtained are shown in the accompanying Figure 1, which is a drawing of the autoradiograph obtained.

The bands observed were separated according to size on the gel, and it was seen that the labelled oligonucleotide probe of formula I had hybridised with the smaller (16S) of the rRNA species of the bacteria, which were selected as representatives of major classes of bacteria. No hybridisation was observed with RNA obtained from mouse liver or from uninfected armadillos. Although faint, a band corresponding to M. leprae was observed for armadillos infected with leprosy.

Claims

1. A DNA probe comprising an oligonucleotide of formula I, II, III

5'-GAGTTGACGTCGTCCCCGCCTTCGTC-3' (I)

5'-GAGGAAGGTGGGGATGACGTCAAGTCATCATG-3' (II)

5'-GTGGACTACTAGGGTATCTAATCCT-3' (III)

5'-ATTCCCCACTGCTGCCTCCCGTAGGAGT-3' (IV)

5'-TTACTCACCCGTTCGCCGCT-3' (V)

or an oligonocleotide complementary to an oligonucleotide of formula I, II, III, IV or V,
or a sub-sequence of 7 or more contiguous bases of an oligonucleotide of formula I, II, III, IV or V, or an oligonucleotide complementary thereto.

2. A DNA probe as claimed in claim 1, which comprises a sub-sequence of 10 or more contiguous bases of an oligonucleotide of formula I, II, III, IV or V, or of an oligonucleotide complementary thereto.

3. A DNA probe as claimed in claim 1, which comprises a sub-sequence of 16 or more contiguous bases of an oligonucleotide of formula I, II, III, IV or V , or of an oligonucleotide complementary thereto.

4. A DNA probe as claimed in any one of claims 1 to 3, wherein the oligonucleotide or sub-sequence thereof has one or more base changes, rearrangements, additions or deletions with the proviso that the resulting DNA probe is capable of hybridising with bacterial nucleic acid material exclusively.

5. A DNA probe as claimed in any one of claims 1 to 4, and which is part of a larger nucleotide with the proviso that the resulting larger nucleotide is capable of hybridising with bacterial nucleic acid material exclusively.

6. A DNA probe as claimed in any one of claims 1 to 5 having a detectable label or marker.

7. A DNA probe as claimed in claim 6, wherein the label is a radioisotope.

8. A DNA probe as claimed in claim 6, wherein the label is biotin.

9. A process for the production of a DNA probe as claimed in any one of claims 1 to 5, which comprises chemically linking the appropriate deoxyribonucleotides as individual base(s) and/or as subsequence(s) of the probe.

10. A process as claimed in claim 9, carried out using solid phase synthesis.

11. A DNA probe as claimed in claim 1, whenever produced by a process as claimed in claim 9 or claim 10.

12. A DNA probe as claimed in any one of claims 1 to 5,

whenever produced by recombinant DNA technology.

13. A method of detecting and/or determining bacteria, which comprises contacting a solid or liquid sample under investigation with a DNA probe as claimed in any one of claims 1 to 8, 11 and 12 under hybridising conditions and detecting and/or determining hybridised DNA probe.

14. A method as claimed in claim 13, wherein the Northern blot method, the Southern blot method or the dot blot method is used to detect and/or determine hybridisation

15. A method as claimed in claim 13 or claim 14, wherein the sample is a solid sample that has been sectioned in a manner suitable for histological examination.

16. A method as claimed in any one of claims 13 to 15, wherein hybridisation is detected and/or determined by a method involving amplification of the label.

17. A method as claimed in claim 13, wherein hybridisation is carried out in solution.

18. A method as claimed in any one of claims 13 to 17, wherein the sample to be investigated is a sample of eukaryotic material.

19. A method as claimed in claim 13, carried out

substantially as described in the Example herein.

20. A kit for the detection and/or determination of bacteria in a solid or liquid sample which comprises

a) a sample of a DNA probe as claimed in any one of claims 1 to 8, 11 and 12,

b) a control sample of bacterial nucleic acid and

c) a control sample of eukaryotic nucleic acid.

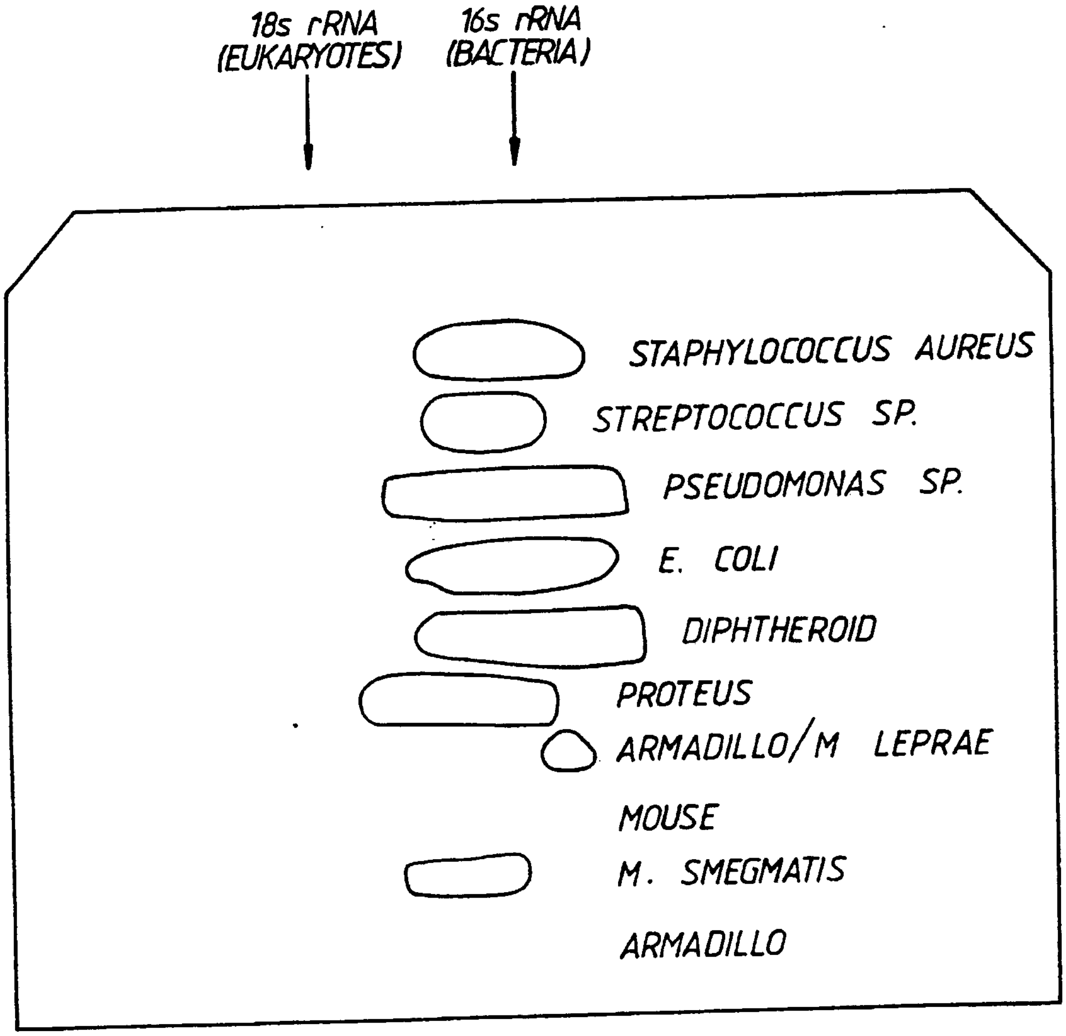
21. A DNA probe as claimed in any one of claims 1 to 8, 11 and 12 for use in the detection and/or determination of bacteria using a hybridisation technique.

22. A DNA probe as claimed in claim 1 that comprises a subsequence of less than 7 contiguous bases of an oligonucleotide of formula I, II, III, IV or V, or of an oligonucleotide complementary thereto, the probe having the ability to hybridise exculsively with bacterial nucleic acid material.

23. An RNA sequence that is complementary to a DNA probe as claimed in any one of claims 1 to 5 or claim 22.

24. A method of detecting and/or determining bacteria, which comprises contacting a solid or liquid sample under

investigation with an RNA probe as claimed in claim 23 under hybridising conditions and detecting and/or determining hybridised RNA probe.


18s rRNA
(EUKARYOTES)
16s rRNA
(BACTERIA)
STAPHYLOCOCCUS AUREUS
STREPTOCOCCUS SP.
PSEUDOMONAS SP.
E. COLI
DIPHTHEROID
PROTEUS
ARMADILLO/M LEPRAE
MOUSE
M. SMEGMATIS
ARMADILLO

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00850

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68 ; C12N15/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q ; C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,8705907 (INSTITUT PASTEUR AND INSTITUT NATIONAL DE LA RECHERCHE MEDICALE) 08 October 1987 see page 6, table see page 16, line 2 - page 18, line 31 ---	1-7, 9-13, 17-19
X	EP,A,0250662 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 07 January 1988 see column 2, line 24 - column 3, line 28 see column 3, line 50 - column 4, line 14 see page 7, lines 23 - 47 ---	1-5, 9-13, 17-19
P	EP,A,0277237 (TORAY INDUSTRIES INC.) 10 August 1988 see front-page, item 87 see page 6, line 10 - page 7, line 8 see page 16, line 2 - page 17, line 2 ---	1-8, 11, 13, 17-19

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 NOVEMBER 1989	19. 01. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 89/00850

SA 30261

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		EP-A-	0245129	11-11-87
		JP-T-	1500001	12-01-89
EP-A-0250662	07-01-88	None		
EP-A-0277237	10-08-88	WO-A-	8800618	28-01-88

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82